Algal Research xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

Cell diameter doesn't affect lipid productivity of *Chlorococcum littorale*

Iago Teles Dominguez Cabanelas [a,b,*,1], Carolina Fernandes [a], Dorinde M.M. Kleinegris [b,1], René H. Wijffels [a,c], Maria J. Barbosa [b,1]

[a] Wageningen University, Bioprocess Engineering, AlgaePARC, P.O. Box 16, 6700 AA Wageningen, Netherlands
[b] Wageningen UR, Food & Biobased Research, AlgaePARC, Bornsesteeg 10, Building 112, 6721NG Bennekom, Netherlands
[c] Faculty Biosciences and Aquaculture, University of Nordland, N-8049 Bodø, Norway

ARTICLE INFO

Article history:
Received 30 September 2015
Received in revised form 31 December 2015
Accepted 6 February 2016
Available online xxxx

Keywords:
Microalgae
Chlorococcum littorale
Cell sorting (FACS)
Cell diameter
Biomass productivity
Lipid productivity

ABSTRACT

We hypothesized that cells with different diameter have different division rates, which could affect lipid productivity (lipid content × biomass productivity). In the present work we assessed the influence of cell diameter, as a sorting parameter, on both biomass and lipid productivity of *Chlorococcum littorale*. Prior to sorting, cells were grown in a batch-wise nitrogen run-out including a long nitrogen depleted phase (N−) to stop cell division, thus only having vegetative cells (Pre-sorting). Cell sorting was done at the end of this N− phase using FACS (fluorescence assisted cell sorting) based on forward scatter as a proxy for diameter (size ranges (μm): 5–6 (small), 8–9 (medium), 11–14 (large) and 5–14 (control)). The sorting was done in 2 pools: multiple-cell (100 cells) and single-cell. After sorting, cells were recovered under low-light for 2 weeks, and used to start the Post-sorting experiment (analogous to Pre-sorting). The populations derived from different sorted pools, single-cell and multiple-cell, showed similar size distributions after re-growth. No difference was observed in biomass and lipid productivities among Post-sorting cells and when compared to Pre-sorting cells under nitrogen depletion. We concluded that cellular size had no effect on both biomass and lipid productivity of *C. littorale*.

1. Introduction

Microalgae have shown potential to replace current feedstock for bulk commodities [11,36]. The positive aspects of microalgae bulk production include sustainability and versatility of the production chain when compared to land crops [10]. Nevertheless, reported biomass and lipid productivities need to be improved to make bulk production economically feasible for commodity products, such as plastics or fuels [26,28].

One approach to increase productivity is to develop more productive strains. This can be done by taking advantage of the natural genetic variability of a parental population to screen and sort cells with different features (e.g. high lipid producers) [22,37]. Another possibility is to increase genetic variability by inducing mutations in a parental population and screen and sort for mutants with abnormal improved features [7,13,32,35]. Fluorescence assisted cell sorting (FACS) is a method which allows rapid screening and sorting of cells with desired characteristics. Sorted cells can be regrown, possibly leading to a new improved culture [13,32,35,37]. FACS can sort cells based on multiple fluorescence and light scattering parameters, which makes it a versatile technology.

Chlorococcum littorale has a high lipid content [29], high photosynthetic efficiency under nitrogen stress [3], and has been used in FACS before [9] and for this reason has been chosen in this study. *C. littorale* is a unicellular microalga whose cell size can range from 5 to 14 μm in the vegetative stage [25]. *C. littorale* can reproduce sexually and asexually by multiple-fission of the mother cell, into 2 to 16 daughter cells (in the shape of spores that can be haploid or diploid, in case of asexual or sexual reproduction, although there is no morphological differences between the two kinds of spores) [25]. The formation of spores is relevant for the sorting, since dividing cells could be mistaken for a large vegetative cell. Up to now the regulation of cell size in microalgae is not completely understood [4,27,33]. Studies with *Chlamydomonas*, a genus with multiple-fission cell division, showed a positive correlation between mother cell's sizes and the number of generated daughters [27]. Other authors have shown that cells might even continue their division without doubling their cell volume. Hence, such cells would produce bigger daughter cells, that would generate more and smaller cells in the next division [4]. Both studies point to a possible influence of cell diameter on the growth patterns of following generations. Up to date, no report has focused on the possible effect of size of vegetative cells on the biomass productivity of bulk cultures. We hypothesized that cells with different diameter have different division rates, which could affect biomass productivity. Hence, differences in biomass productivity

* Corresponding author at: Wageningen University, Bioprocess Engineering, AlgaePARC, P.O. Box 16, 6700 AA Wageningen, Netherlands.
E-mail address: iago.dominguezteles@wur.nl (I.T.D. Cabanelas).
[1] www.AlgaePARC.com.

http://dx.doi.org/10.1016/j.algal.2016.02.002
2211-9264/

could consequentially affect lipid productivity (lipid content × biomass productivity).

In the present work we assessed the influence of cell diameter, as sorting criterion, on both biomass and lipid productivity of vegetative cells of *C. littorale*. *C. littorale* was grown in a batch-wise nitrogen run-out followed by a long nitrogen-depleted phase to stop cell division and to induce lipid accumulation, thus having only vegetative, lipid-rich cells. At the end of the nitrogen-depleted phase (15 days, after cell size was stable) cells were sorted in four groups of different diameters: small (5–6 μm), medium (8–9 μm), large (11–14 μm) and a control (5–14 μm). We compared both biomass and lipid productivities of sorted cells of *C. littorale* with the growth of the parental population. Additionally, we compared the daily differences in cell size distribution, photosystem II quantum yield, autofluorescence and intra-cellular lipid fluorescence.

2. Materials and methods

2.1. Inoculum preparation, cultivation and culture screening with FACS

Inoculum of *C. littorale* (NBRC 102761) was prepared from samples preserved under low light conditions (16 μmol m^{-2} s^{-1}) in borosilicate tubes containing growth medium and agar (12 g l^{-1}). Small samples were transferred from agar to 200 ml sterile borosilicate Erlenmeyer flasks, containing 100 ml of sterile growth medium. *C. littorale* was grown in salt water-like medium with the following composition (g l^{-1}): NaCl 24.55, $MgSO_4 \cdot 7H_2O$ 6.60, $MgCl_2 \cdot 6H_2O$ 5.60, $CaCl_2 \cdot 2H_2O$ 1.50, $NaNO_3$ 1.70, HEPES 11.92, $NaHCO_3$ 0.84, EDTA-Fe(III) 4.28, K_2HPO_4 0.13, KH_2PO_4 0.04. The medium also contained the following trace elements (mg l^{-1}): $Na_2EDTA \cdot 2H_2O$ 0.19, $ZnSO_4 \cdot 7H_2O$ 0.022, $CoCl \cdot 6H_2O$ 0.01, $MnCl_2 \cdot 2H_2O$ 0.148, $Na_2MoO_4 \cdot 2H_2O$ 0.06, $CuSO_4 \cdot 5H_2O$ 0.01.

The sequence of experiments performed in this work is represented in Fig. 1. After inoculum preparation we set-up a preliminary test to determine the boundaries for the sorting gates based on cell size using fluorescence assisted cell sorting (FACS). The preliminary test was performed before but analogous to the experiment explained in the next section.

2.2. Experimental set-up

The experimental set-up was the same for both Pre-sorting and Post-sorting experiments (Fig. 1). Experiments were inoculated with an initial OD_{750} value of 0.5 (~0.7 g DW l^{-1}). The algae were cultivated in 200 ml borosilicate Erlenmeyer flasks (100 ml medium per flask) and kept in an Infors Multitron Pro orbital shaker incubator. Growth conditions were: 25 ± 0.2 °C, 120 rpm, 60% humidity, 2% CO_2 enrichment on air, and 120 ± 2 μmol m^{-2} s^{-1} continuous light (24 h).

Both experiments had 2 phases: a growth phase (N+) and a nitrogen depleted (N−) phase. During the growth phase nitrogen (N–NO_3) was allowed to run-out progressively (at day 5 NO_3 was completely consumed by the cells). After reaching stationary phase, the cultures were diluted 5× to a biomass concentration of 1.4 ± 0.1 DW g l^{-1}, in sterile medium without NO_3^-, to allow further light penetration in the culture and enhance lipid production. After dilution, cells were monitored until maximum size was achieved, and was constant for 3 days (after 15 days from start experiment). All daily measurements were done at the same time every day and are described in the following section.

Multiple-cell sorting (3 × 100 cells) was done based on cell size in four groups, at the end of the stationary phase (Fig. 3). After sorting, cells were re-suspended in 50 ml sterile falcon tubes containing fresh and sterile nitrogen-replete medium into. Cells grew for 2 weeks in the tubes under low light conditions (16 μmol m^{-2} s^{-1}). After this, cells were transferred to sterile borosilicate Erlenmeyer's, where Post-sorting experiments were conducted under similar conditions as the Pre-sorting experiments and with the same daily measurements.

Additionally single-cells were sorted from the same parental population on the same day (Fig. 1). Fifteen replicates per group of single-sorted cells were taken to increase sample size and to assure reproducibility of Post-sorting results. Single cell sorting was done analogous to and immediately after the multiple-cell sorting. After 18 days of growth under low-light conditions (16 μmol m^{-2} s^{-1}) the sorted cells were centrifuged at 1224 ×*g* for 10 min, washed with sterile medium and readily analyzed for size and autofluorescence using the FlowCAM® fluid imaging system (settings are presented in the next section).

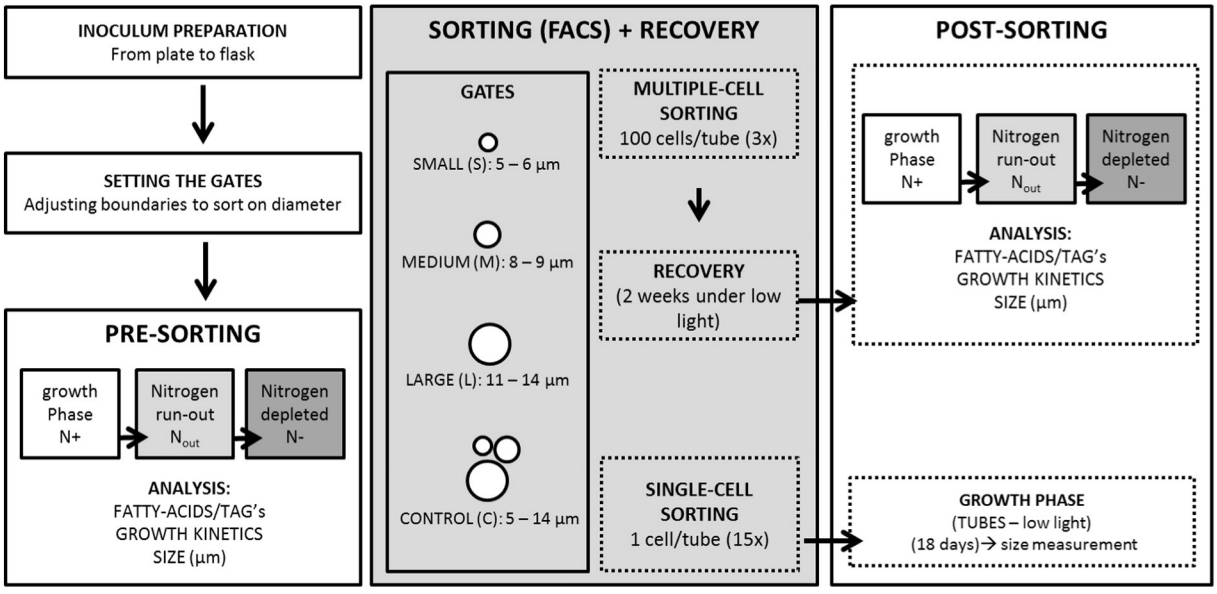

Fig. 1. Experimental flow diagram followed in this work. Inoculum preparation: fresh cultures were prepared from agar plate's cultures and kept under controlled conditions. Setting the gates: a preliminary test was done to set the boundaries of the gates used for the actual sorting. Pre-sorting: Batch nitrogen run-out cultivations were done in flasks including a growth phase (N+, in white), a nitrogen run-out (N_{out}, in light gray) and a nitrogen depleted phase (N−, in dark gray). The same colors were used in Figs. 4 and 7. Sorting: after cell size was stabilized during nitrogen depletion (3 days with the same size distribution) the multiple-cell sorting was done with 4 different gates based on size (3 × 100 cells/replicate). Post-sorting: experiments analogous to Pre-sorting to compare biomass and lipid productivities; the single-cell sorted cells were readily analyzed after 18 days of re-grow. Acronyms: FACS (fluorescence assisted cell sorting) and TAG's (triacylglycerides).

Please cite this article as: I.T.D. Cabanelas, et al., Cell diameter doesn't affect lipid productivity of *Chlorococcum littorale*, Algal Res. (2016), http://dx.doi.org/10.1016/j.algal.2016.02.002